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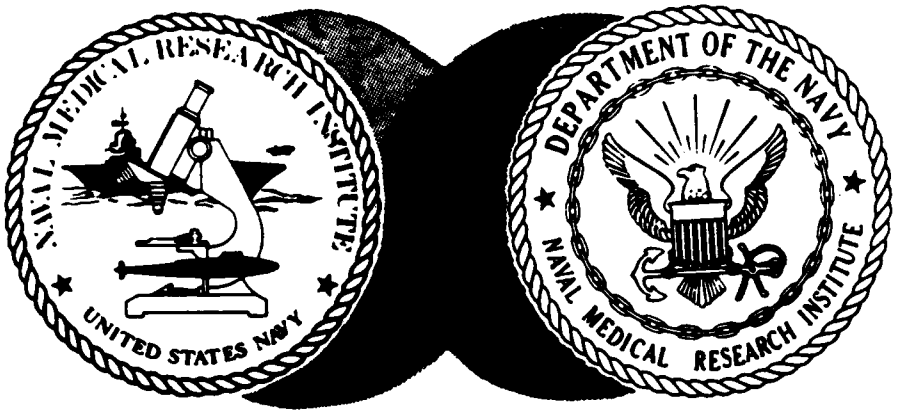

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B LYMPHOCYTE SUBPOPULATION DEFINED BY A RAT MONOCLONAL ANTIBODY, 14G8

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14G8 is monoclonal rat antibody that recognizes an antigen found on 30 to 40% of B lymphocytes from normal mice and on approximately 65% of B lymphocytes from mice with the *xid*-determined immune defect. 14G8$^+$ B cells from normal mice resemble B cells from mice with the *xid*-determined defect in that the median amount of membrane IgM expressed per cell is much larger than that of 14G8$^-$ B cells. The frequency of 14G8$^+$ cells is highest in neonatal mice (~55% of all spleen cells) and falls with age to ~25% of all spleen cells in adult mice. Relatively few lymph node or bone marrow B cells express the antigen recognized by 14G8. 14G8 also reacts with 50% of resident peritoneal cells and with red blood cells. 14G8$^+$ and 14G8$^-$ B cell preparations were obtained by fluorescence-activated cell sorting and by adherence to 14G8 coated dishes. 14G8$^+$ cells responded with *in vitro* proliferation to both anti-μ and to LPS. Cell cycle analysis indicated that approximately 33% of these cells entered S phase in response to LPS and 38% in response to anti-μ. In contrast, 14G8$^-$ cells responded poorly to LPS (7% of cells entered S phase) although they showed good responses to anti-μ (40% of cells entered S phase). Thus, 14G8$^+$ B cells, despite their similarity to B cells from mice with the *xid* defect, can proliferate to anti-μ, which B cells from defective mice fail to do. 14G8 provides a monoclonal antibody valuable in the description of functional B cell subpopulations.

B lymphocytes of mice may be divided into subpopulations distinguishable from one another on the basis of functional properties and expression of membrane antigens (1, 2). In particular, one subset of B cells expresses the Lyb-3 (3) and Lyb-5 (4) alloantigens and appears to be responsible for the capacity of mice to produce antibodies upon immunization with a group of antigens, designated type II thymus-independent (TI-2) antigens (5–8), and for the ability of B lymphocyte populations to proliferate *in vitro* to anti-immunoglobulin antibodies (9). This B lymphocyte subpopulation is absent from the mutant CBA/N mouse (5), that expresses the *xid*-determined defect in B lymphocyte function. No *distinctive* markers or functions of the other major B lymphocyte subpopulation have yet been established, although these cells can respond to type I TI antigens (10–12) and to many thymus-dependent antigens. Singer *et al.* (13) have recently suggested that these cells are uniquely responsible for histocompatibility-restricted T cell-B cell collaboration.

The antibodies that distinguish these populations, anti-Lyb-3 and anti-Lyb-5, are limited in their utility because they are difficult to prepare. Furthermore, anti-Lyb-3 does not fix complement and anti-Lyb-5 requires extensive absorption and testing. Consequently, we undertook an effort to produce monoclonal antibodies that recognized B lymphocyte subpopulations. In this paper, we report the description of a rat monoclonal antibody, 14G8, which identifies an antigen found on a subpopulation of B cells that comprises 30 to 40% of normal B cells and approximately 65% of B cells from mice with the *xid*-determined defect. Certain of the functional properties of cells expressing and lacking this membrane antigen will be described.

MATERIALS AND METHODS

Animals. DBA/2N, (DBA/2N × CBA/N)F_1 (DCF$_1$) ♂, (CBA/N × DBA/2N)F_1 (CDF$_1$) ♂ mice, and Fisher rats were obtained from the Small Animal Section, Division of Research Services, National Institutes of Health, Bethesda, MD. SJL/J mice were purchased from the Jackson Laboratory, Bar Harbor, ME.

Mitogens. Lipopolysaccharide W (LPS), *E. coli* 0111:B4, was obtained from Difco Laboratories, Detroit, MI. Affinity purified goat anti-μ antibodies were kindly provided by Dr. Anthony DeFranco (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases [NIAID], Bethesda, MD). The goat anti-μ antiserum from which the specifically purified antibodies were prepared was generously supplied by Dr. Richard Asofsky (Laboratory of Microbial Immunity, NIAID).

Enumeration of B cells. B cells were enumerated in all experiments with Bet 1 (14), a monoclonal rat anti-mouse IgM antibody. Briefly, splenic B cells were detected by either incubation with Bet 1 hybridoma supernatant followed by fluorescein-conjugated SJL anti-rat IgG or by incubation with biotin-conjugated Bet 1 followed by XRITC (a substituted rhodamine isothiocyanate)-conjugated avidin (Vector Laboratories, Inc., Burlingame, CA).

Production and screening of hybridomas secreting monoclonal rat anti-mouse B cell antibodies. Production of rat × mouse hybridomas has been previously described by Springer *et al.* (15) and Ledbetter and Herzenberg (16). Fisher rats were immunized i.p. and boosted 3 wk later with 10×10^6 DBA/2 spleen cells in Hanks' balanced salt solution (HBSS). Three days after boosting, spleen cells of the immunized rat were fused with cells of the hypoxanthine-guanine phosphoribosyl transferase-negative NS-1 myeloma line derived by Köhler *et al.* (17). Fifty percent polyethyleneglycol 1500 (Fisher Scientific Company, Fairlawn, NJ) was used for fusions; details of fusion procedures and hypoxanthine-aminopterin-thymidine (HAT) selection were as published by Oi and Herzenberg (18). Three to 4 wk after the fusion, culture supernatants were screened for their ability to bind to DBA/2 spleen cells. The screening procedure involved the reaction of 10^6 DBA/2 spleen cells with 50 μl of hybridoma supernatant followed by a fluorescein-conjugated mouse anti-rat IgG and subsequent analysis on a fluorescence-activated cell sorter (FACS II, Becton-Dickinson, Mountain View, CA).

Production of mouse anti-rat IgG antisera. Mouse anti-rat IgG antisera were collected from SJL/J mice immunized with purified rat IgG as previously described (14).

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Purification of monoclonal rat antibodies. 14G8 and monoclonal rat anti-TNP antibodies were purified from culture supernatants by affinity chromatography using a monoclonal mouse anti-rat IgG1,2a column as previously described (14). Hybridoma cells producing monoclonal rat anti-TNP antibody were kindly provided by Drs. Joseph Davie and Jeff Paslay, Washington University, St. Louis, MO.

Determination of heavy chain class of monoclonal rat antibodies. Supernatant from hybridoma cultures was concentrated 10-fold using a Minicon concentrator (Amicon, Lexington, MA). The concentrated supernatant was analyzed with class-specific anti-rat Ig antibodies (Miles Laboratories, Elkhart, IN) by Ouchterlony immunodiffusion. 14G8 was identified as rat IgG2a by this method. The monoclonal rat anti-TNP antibody also belongs to the IgG2a subclass (personal communication, Dr. Joseph Davie).

Preparation of fluorescent antibodies. Purified 14G8 and anti-TNP monoclonal antibodies in phosphate-buffered saline (PBS) were adjusted to pH 9.5 with 5% Na_2CO_3. The protein concentration was usually 2 to 3 mg/ml. Fluorescein-isothiocyanate (FITC) was mixed with Celite (Calbiochem, La Jolla, CA) in a ratio of 1:10 (w/w). Two parts of protein were mixed with 1 part of FITC:Celite (w/w) and reacted at room temperature for 20 to 60 min. Unreacted FITC was separated from conjugated protein by filtration through a Sephadex G-25 column. The fluorescein/protein (F/P) ratio was 3.0 for both fluoresceinated 14G8 and anti-TNP. Florescein conjugated 14G8 and anti-TNP were ultracentrifuged for 1 hour at 100,000 × G to remove aggregated immunoglobulins.

SJL anti-rat IgG2 antisera were precipitated with 33% ammonium sulfate, redissolved, dialyzed against PBS, and conjugated with FITC according to the above procedure.

Preparation of biotin-conjugated monoclonal antibodies. Biotin-conjugated monoclonal antibodies were made by reacting 1 mg of purified monoclonal antibody in 0.1 M $NaHCO_3$, pH 8.4, with 120 μg of biotin succinimide (Biosearch Research Biochemicals, San Rafael, CA) according to Bayer and Wilcheck (19).

Preparation of spleen cells for staining with fluorescein-conjugated antibodies. Spleens were removed from mice and gently teased apart in HBSS with forceps to make single cell suspensions. Erythrocytes were removed by NH_4Cl-erythrocyte lysing buffer (20). After lysis of erythrocytes, dead cells were removed by low ionic strength, iso-osmotic medium according to von Boehmer and Shortman (21). The cells were made up to 20 × 10^6/ml in HBSS containing 1% bovine serum albumin (BSA) and 0.1% NaN_3.

Fluorescence analysis on a fluorescence-activated cell sorter. For single parameter fluorescence analysis, 50 μl (10^5) of cells were either incubated with appropriate fluorescein-conjugated monoclonal antibodies or incubated with 10 to 50 μl of hybridoma supernatant, followed by reaction with a previously titrated quantity of fluorescein-conjugated SJL anti-rat IgG. All incubation steps were carried out on ice for 20 to 30 min, followed by 2 washes with HBSS containing 1% BSA and 0.1% NaN_3. The stained cells were then analyzed by flow microfluorometry (FMF) on a fluorescence-activated cell sorter. Basic principles of FMF analysis have been described by Loken and Herzenberg (22) and the conditions of analysis were as reported by Sharrow *et al.* (23). The histograms of fluorescence distribution shown in *Results* were obtained by analyzing 50,000 cells per sample and are always plotted as number of cells (*y*-axis) *vs* fluorescence units (*x*-axis, that contained 1000 channels). Light scatter gating was employed to limit the analysis to viable cells. Macrophages were probably included in this population but dead cells and any erythrocytes remaining after NH_4Cl lysis were excluded.

For dual parameter fluorescence analysis, cells were incubated with fluorescein-conjugated monoclonal rat antibody and biotin-conjugated Bet 1, followed by reaction with a previously titrated amount of XRITC-avidin. The stained cells were then analyzed using a FACS II dual laser system. Conditions for dual laser setup, operation, and details for data collection and analysis have been published by Segal *et al.* (24). Briefly, the data were collected and stored in a PDP11/40 computer as a 64 × 64 channel matrix for correlation of dual color fluorescence. Results of dual color fluorescence analysis shown in *Results* were obtained by performing slice analyses with the aid of a computer. By arbitrarily choosing a "fluorescence 1" window, the computer is asked to generate a plot of cell number *vs* "fluorescence 2" intensity, disregarding all cells with fluorescence 1 intensities falling outside the chosen window. Such "slice" plots are shown in Figures 2, 4, and 5; in these plots, the *x*-axis is made up of 64 channels. The *y*-axis (number of cells) has been normalized such that the area under the curve is equivalent to that generated when 50,000 cells are analyzed.

Calculation of percentage of positive cells by fluorescence analysis on FACS. Positive cells were defined as cells with fluorescence intensities greater than the point at which the fluorescence histogram of stained cells could be clearly distinguished from that of control cells. The percentage of cells with specific staining was then calculated as follows:

$$\% \text{ Specific positives} = \frac{\% \text{ Positive stained cells} - \% \text{ positive control cells}}{100 - \% \text{ positive control cells}} \times 100$$

Isolation of 14G8⁺ and 14G8⁻ B cells by cell sorting. DCF_1 δ spleen cells, that had been treated with anti-Thy-1.2 (New England Nuclear, Boston, MA) and complement, were stained with fluorescein-conjugated 14G8 antibody. The brightest 10% of cells stained by 14G8 were sorted as "14G8 bright" cells, and the least fluorescent 40% of cells were sorted as "14G8⁻" cells. Reanalysis of sorted cells showed at least 85% of "14G8 bright" cells were 14G8⁺ and that less than 5% of "14G8⁻" cells were 14G8⁺.

Fractionation of B cells using tissue culture dishes sensitized with 14G8 monoclonal antibody. Plate fractionation procedures employed were a modification of those reported by Mage *et al.* (25). Individual tissue culture dishes (100 × 20 mm, Corning 25020) were sensitized for 1 hr at room temperature with 5 ml of a 20 μg/ml solution of purified K2530, a monoclonal mouse anti-rat IgG1,2a antibody (14) in PBS.

The dishes were washed 5 times with PBS, and 5 ml of 14G8 hybridoma supernatant was allowed to bind to such K2530-coated dishes overnight. The plates were again washed 5 times with PBS immediately before addition of cells. 75 to 80 × 10^6 spleen cells in 5 ml of HBSS containing 20% FCS and 0.1% NaN_3 were allowed to adhere to 14G8-sensitized dishes at room temperature. After 45 min, nonadherent cells were transferred with a Pasteur pipet to another 14G8-sensitized dish, and incubation was continued for an additional 60 min. Nonadherent cells obtained after 2 cycles of incubation on 14G8-dishes are referred to as "14G8-depleted" B cells.

Cells adherent to the first set of 14G8 plates were gently rinsed 10 times with HBSS + 20% FCS + 0.1% NaN_3. The remaining adherent cells were removed by pipeting. These cells are referred to as "14G8-enriched" B cells.

Cell culture and ^{3}H-thymidine incorporation. Spleen cells were cultured in a final vol of 0.2 ml in Mishell-Dutton medium (26) containing 10% fetal calf serum (Rehatiun, Armour Pharmaceutical Co., Pheonix, AZ), 16 mM HEPES[2] buffer and 5 × 10^{-5} M 2-merceptoethanol in 96-well tissue culture clusters (No. 3596, Costar, Division of Data Packaging Corporation, Cambridge, MA). All cultures were pulsed with 1 μCi of methyl-^{3}H-thymidine (6.7 Ci/mmol, New England Nuclear) at specific times after initiation of cultures and harvested 4 hr later using a MASH harvester (Microbiological Associates, Walkersville, MD). Liquid scintillation fluid was added to the filters, and radioactivity was determined in a Beckman liquid scintillation counter.

Cell cycle analysis of mitogen-stimulated cells. Colcemid (GIBCO Laboratories, Grand Island, NY) was added to all cultures to a final concentration of 25 ng/ml 1 day after initiation of cultures, to block cell division. Determination of DNA content was performed at indicated times by staining with propidium iodide (27) and analysis on a TPS I cell sorter (Coulter Electronics, Inc., Hialeah, FL). Percentages of cells in G_1, S, and G_2+M phases of the cell cycle were determined using a mathematical algorithm according to Ravache *et al.* (28).

RESULTS

14G8 reacts with a subpopulation of adult spleen cells. Figure 1 shows fluorescence profiles of DCF_1 δ spleen cells reacted with culture supernatant from 14G8-producing cells followed by fluoresceinated mouse anti-rat IgG. Profiles displayed in panels *A* and *B* were generated by using linear and logarithmic amplifiers, respectively, for FMF analysis. The number of cells specifically stained by 14G8 was determined as described in *Materials and Methods*. 14G8 specifically reacted with 25.2% of spleen cells, based on analysis using the linear amplifier, and with 27.7% of spleen cells, based on logarithmic analysis. Neither the number of positive cells nor the intensity of fluorescence of positive cells increased with the use of increasing amounts of 14G8, which suggested that we have saturated the antigenic sites on positive cells. Thus, the low intensity of staining indicates the existence of a small number of antigen molecules on the positive cells rather than low affinity binding by 14G8. The fluorescence profiles of cells incubated with a control culture supernatant obtained from hybridoma cells secreting a rat IgG2a anti-TNP monoclonal antibody followed by fluoresceinated SJL anti-rat IgG was indistinguishable from profiles obtained with fluoresceinated SJL anti-rat IgG alone. Since 14G8 is a rat IgG2a immunoglob-

[2] Abbreviations used in this paper: DCF_1, $(DBA/2 \times CBA/N)F_1$; CDF_1, $(DBA/N \times DBA/2)F_1$; FMF, flow microfluorometry; TI, thymus independent; HAT, hypoxanthine-aminopterin-thymidine; HEPES, *N*-2-hydroxyethyl piperazine-*N*'-2-ethanesulfonic acid; F/P ratio, fluorescein/protein ratio.

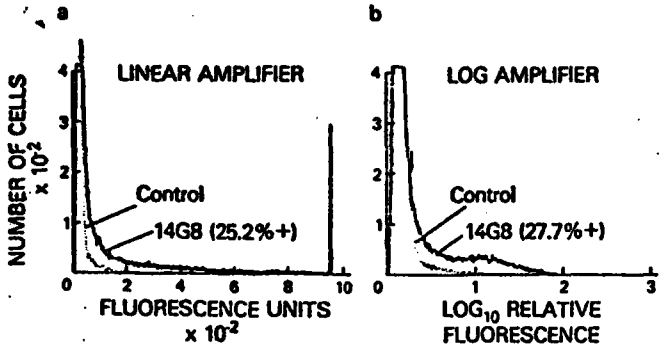

Figure 1. 14G8 reacts with a subpopulation of spleen cells. Spleen cells were incubated with 14G8 hybridoma supernatant followed by fluorescein-conjugated SJL anti-rat IgG antibody. FMF analysis were then performed with the use of either a linear or a logarithmic amplifier. Control cells were incubated with fluorescein-conjugated SJL anti-rat IgG antibody only.

ulin, this indicates the specificity of its binding. To further verify the specificity of 14G8 staining, we compared direct staining of spleen cells with fluorescein conjugates of 14G8 and anti-TNP control antibodies. Both reagents were affinity purified by adsorption to a K2530-Sepharose column (a monoclonal mouse anti-rat IgG2a column) and subsequent elution with 3.5 M $MgCl_2$. Both purified antibodies were conjugated with fluorescein isothiocyanate to an F/P ratio of 3.0 and ultracentrifuged for 1 hr at 100,000 × G to remove aggregated immunoglobulins. DCF_1 δ spleen cells were stained with either 1 μg of fluorescein-conjugated 14G8 or 1 μg of fluorescein-conjugated anti-TNP antibody per 10^5 cells. Positive cells are arbitrarily defined as cells with fluorescence intensities higher than the first point at which the fluorescence histogram of 14G8 stained cells starts to be clearly distinguishable from that of unstained cells. Using this method for scoring positive cells and without any subtraction of control values, there were 16.0%, 3.6%, and 1.9% positive cells in 14G8 stained, anti-TNP stained, and unstained cells, respectively, in this experiment.

14G8 reacts with a subpopulation of B cells. The relationship between the presence of surface IgM and the expression of the determinant recognized by 14G8 was studied by reacting DCF_1 δ spleen cells with fluorescein-conjugated 14G8 antibody and with biotin-conjugated monoclonal rat anti-mouse IgM antibody followed by XRITC-conjugated avidin (Fig. 2). By defining cells in the shaded area on the fluorescence histogram as positive, 16.4% of the spleen cells were $14G8^+$ (panel *A*) and 50.5% were IgM^+ (panel *D*). Arrows indicate the median fluorescence intensity of positive cells. An analysis limited to those cells that were IgM^+ indicated that 28% were $14G8^+$ (panel *B*), and a similar analysis of the IgM^- cells showed only 1.5% to be $14G8^+$ (panel *C*). When $14G8^+$ cells were analyzed, at least 90% were IgM^+ (panel *E*). Of the $14G8^-$ spleen cells, 43% were IgM^+ (panel *F*). These results indicate that almost all $14G8^+$ cells are IgM^+ but that only a portion (about 30%) of IgM^+ cells express the determinant recognized by 14G8.

A further difference between $14G8^+IgM^+$ and $14G8^-IgM^+$ cells may be seen by inspection of the fluorescence histograms of the anti-IgM staining of these populations (panels *E* and *F*). $14G8^+IgM^+$ cells have a median IgM fluorescence intensity approximately twice that of $14G8^-IgM^+$ cells. In addition, the $14G8^+IgM^+$ cells are considerably more heterogeneous than $14G8^-IgM^+$ cells in the amount of IgM they express.

14G8 reacts with more CDF_1 δ spleen cells than DCF_1 δ spleen cells. Figure 3 compares fluorescence profiles of phenotypically normal DCF_1 δ spleen cells with those of an immune defective CDF_1 δ reacted with either 14G8 or a rat monoclonal anti-mouse IgM hybridoma supernatant followed by fluorescein-conjugated mouse anti-rat IgG. Control cells were treated with fluorescein-conjugated mouse anti-rat IgG only. By defining cells to the right of the arrow as positive, there were 63% and 43% IgM^+ cells in DCF_1 δ and CDF_1 δ spleen cells, respectively. These values were arrived at after appropriate subtraction of control values as described in *Materials and*

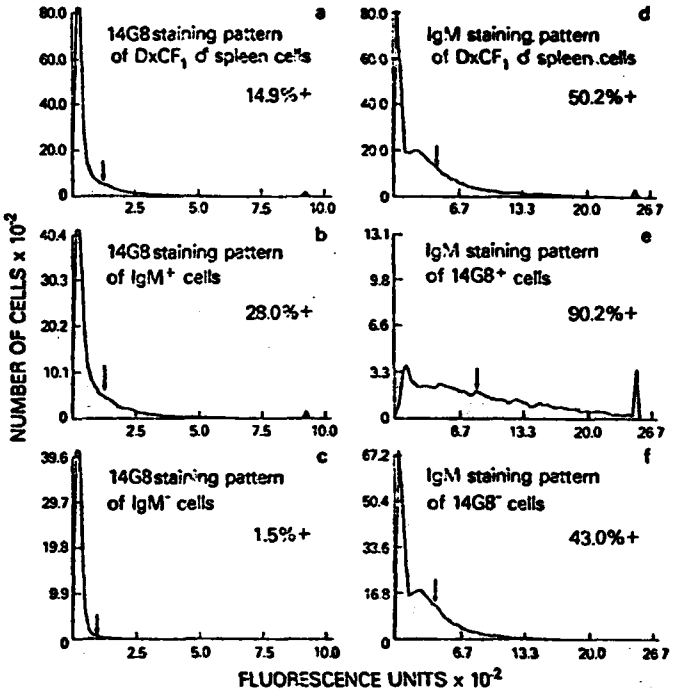

Figure 2. Dual fluorescence analysis of DCF_1 δ spleen cells with 14G8 and anti-IgM monoclonal antibodies. Spleen cells from DCF_1 δ mice were incubated simultaneously with fluorescein-conjugated 14G8 and biotin-conjugated monoclonal anti-IgM followed by XRITC-avidin. They were then subjected to dual fluorescence FMF analysis as described in *Materials and Methods*. Arrows indicate median fluorescence of positive cells, determined as described in *Materials and Methods*. Dual parameter analysis of CDF_1 δ spleen cells (Fig. 4) was performed on the same day.

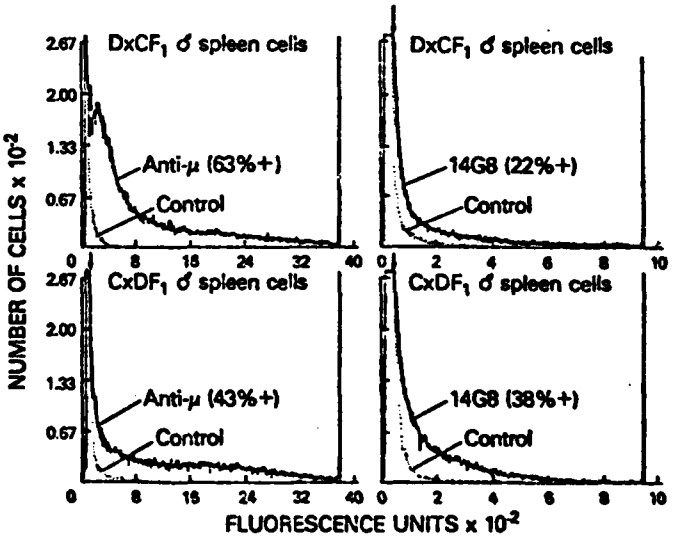

Figure 3. Fluorescence histograms of normal and CBA/N spleen cells with anti-IgM and 14G8 monoclonal antibodies. Spleen cells from phenotypically normal (DCF_1 δ) or defective (CDF_1 δ) mice were incubated with 14G8 or anti-IgM hybridoma supernatant followed by fluorescein-conjugated SJL anti-rat IgG. Control cells received only fluoresceinated SJL anti-rat IgG.

Methods. The IgM staining profiles confirm results published by Scher *et al.* (29) in showing relatively few cells bearing low amounts of surface IgM in CDF_1 δ mice. This results in a higher than normal median fluorescence intensity and a greater heterogeneity of IgM expression on their B cells. Even though the percentage of B cells in DCF_1 δ spleen was approximately one and one-half times that of CDF_1 δ spleen, there were more cells bearing the determinant recognized by 14G8 in CDF_1 δ spleen than in DCF_1 δ spleen (38% *vs* 22%). The 14G8 staining patterns of DCF_1 δ and CDF_1 δ spleen cells were similar despite differences in percentage of $14G8^+$ cells and differences in IgM staining patterns of these cells.

These results suggest that a much larger fraction of IgM^+ cells in mice with *xid*-determined immune defect express the antigen recognized by 14G8 than do IgM^+ cells from phenotypically normal mice.

14G8 reacts with a major subpopulation of CDF_1 δ B cells. To directly examine the expression of the determinant recognized by 14G8 and of IgM on spleen cells from mice with the *xid*-determined immune defect, CDF_1 δ spleen cells were reacted with fluorescein-conjugated 14G8 and with a biotin-conjugated monoclonal rat anti-mouse IgM, followed by XRITC-avidin (Fig. 4). There were 28% $14G8^+$ cells (panel *A*) and 39% IgM^+ cells (panel *D*). Of the IgM^+ cells, 63% were $14G8^+$ (panel *B*), whereas there were only 5% $14G8^+$ cells among IgM^- cells (panel *C*). Of $14G8^+$ cells, at least 83% were IgM^+, whereas only 20% of $14G8^-$ cells were IgM^+ (panels *E* and *F*). The $14G8^+IgM^+$ CDF_1 δ spleen cells display a median IgM fluorescence intensity and fluorescence profile, which is similar to that of $14G8^+IgM^+$ cells from normal mice (Fig. 2) and to unseparated IgM^+ spleen cells from genetically defective CDF_1 δ mice (Fig. 4). This pattern is distinctly different from that of the total population of IgM^+ cells from phenotypically normal

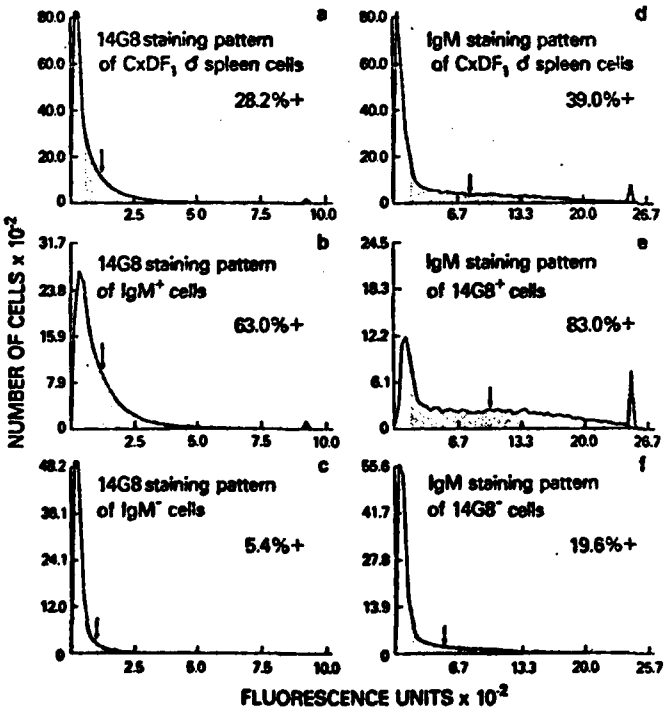

Figure 4. Dual fluorescence analysis of CDF_1 δ spleen cells with 14G8 and anti-IgM monoclonal antibodies. Spleen cells from CDF_1 δ mice were incubated simultaneously with fluorescein-conjugated 14G8 and biotin-conjugated monoclonal anti-IgM followed by XRITC-avidin. They were then subjected to dual fluorescence FMF analysis. Arrows indicate median fluorescence of positive cells. Dual parameter analysis of DCF_1 δ spleen cells (Fig. 2) was performed on the same day.

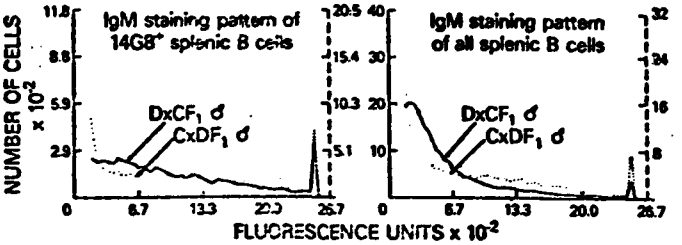

Figure 5. IgM-staining pattern of $14G8^+$ B cells and all B cells. IgM-staining pattern of all B cells and of "$14G8^-$" B cells from normal (DCF_1 δ) and defective (CDF_1 δ) mice are shown. The "slices" are generated as described in *Materials and Methods*.

TABLE I

Tissue distribution of cells expressing the determinant recognized by 14G8

Tissue[a]	n[b]	% B Cells[c]	% Cells Bound[c] by 14G8
Spleen	7	54.2 ± 4.3	22.2 ± 4.0
Lymph node	2	9.5 ± 0.3	1.0 ± 0.1
Bone marrow	1	19.0	2.8
Thymus	1	0.5	0.7

[a] Cells from indicated tissues were obtained from DCF_1 δ mice 2 to 5 mos of age.

[b] n = number of determinations.

[c] Percentages of B cells and cells bound by 14G8 were determined by FMF after the cells were incubated with either rat monoclonal anti-IgM hybridoma supernatant or 14G8 hybridoma supernatant, respectively, followed by fluorescein-conjugated SJL anti-rat IgG. Average values ± SD are shown.

DCF_1 δ mice (Fig. 2). Figure 5 illustrates these points by presenting the positive portions of these patterns adjusted so that there are equal areas under each curve.

Tissue distribution of cells recognized by 14G8 monoclonal antibody. Data presented in Table I show that the percentage of cells bearing the determinant recognized by 14G8 in spleen is approximately 40 to 50% that of IgM^+ cells. Bone marrow contained 19.0% IgM^+ cells and very few (2.8%) cells bound by 14G8. Therefore, the majority of bone marrow B cells must be $14G8^-$.

Thymocytes contained less than 1% of IgM^+ cells as well as cells recognized by 14G8. In 2 experiments, lymph node cells contained approximately 9 to 10% B cells but only 1% $14G8^+$ cells. In this respect, lymph node B cells are similar to bone marrow B cells in that the great majority of them are not recognized by 14G8. In addition to the reactivity of 14G8 with a subpopulation of B cells, it also recognizes a determinant on erythrocytes, since 14G8 hybridoma supernatant causes hemagglutination at as low as a 1:1024 dilution. Further analysis showed that 14G8 also binds to approximately 50% of resident peritoneal cells (data not shown). These cells are most consistent with macrophages since fluorescence *vs* light scatter dual parameter analysis showed that the peritoneal cells recognized by 14G8 are large cells with a high level of autofluorescence.

Ontogeny of cells expressing the determinant recognized by 14G8 in spleen. Spleen cells from DCF_1 δ mice from 1 wk to 6 mo of age were examined for percentage of B cells and cells expressing the determinant recognized by 14G8. Table II shows that there were in general 50 to 60% IgM^+ cells among spleen cells of mice of all ages tested, whereas the percentage of cells expressing the determinant recognized by 14G8 steadily decreased from 55% at 1 wk of age to 27% at 6 wk of life and then remained constant around 20 to 25% (9 wk to 6 mo).

Mitogen responses of sorted "14G8 bright" and "$14G8^-$" B cells. B cells from normal mice that express the determinant recognized by 14G8 resemble B cells from mice with the *xid*-determined immune defect in their surface IgM expression, and cells expressing the determinant recognized by 14G8 are

enriched among the B cells of defective mice. Consequently, we wished to examine functional properties of normal cells expressing or lacking the determinant recognized by 14G8 in terms of known functional properties of cells from mice with the *xid*-determined defect. The simplest system to examine is the proliferative response to mitogens. B cells from defective mice fail to proliferate in response to anti-μ. Furthermore, they do respond to LPS, although at low cell densities their responses tend to be poorer than those of normal controls.

Anti-Thy-1.2 + C treated DCF_1 δ spleen cells were stained with fluorescein-conjugated 14G8 and sorted according to the amount of bound 14G8. Since B cells express relatively few molecules bearing the determinant specifically recognized by 14G8, "high gains" on the cell sorter are usually selected for enumeration of cells expressing the determinant recognized by 14G8 and for the sorting of these cells. This results in a relatively high background fluorescence of control cells; thus, approximately 10 to 20% of cells scored as $14G8^+$ could be accounted for by unstained cells.

To minimize the potential contamination of $14G8^+$ cells with negative cells, we set windows on the cell sorter to sort only the 10% brightest cells into the "14G8 bright" fraction. "$14G8^-$" cells were made up of the 40% of cells displaying the lowest fluorescence intensities. When reexamined after sorting, the negative cells were indistinguishable from unstained cells and the "14G8 bright" cells generally contained 90% positive cells.

Microcultures containing 50,000 sorted cells were established with either LPS or anti-μ as mitogens. Because proliferative responses stimulated by anti-μ are quite density dependent when calculated on a per cell basis and because of the possibility that $14G8^-$ cells might be depleted of macrophages, CDF_1 δ spleen cells (2×10^5) were added as fillers to cultures in which the effect of anti-μ was to be tested. Cultures containing only CDF_1 δ spleen cells do not incorporate ^{3}H-thymidine in response to anti-μ. Furthermore, when CDF_1 δ spleen cells are used as fillers, a linear relation between number of anti-Thy-1.2 + C treated DCF_1 δ spleen cells added and ^{3}H-thymidine uptake in response to anti-μ was observed over cell densities ranging from 40,000 to 200,000 per well (data not shown). Unsorted cells and the "$14G8^-$" cells responded to anti-μ to a comparable degree. In 2 of 3 experiments "14G8 bright" cells responded to anti-μ, although in both of these cases the magnitude of their response was somewhat less than that of "14G8 negative" B cells.

The most striking functional difference between "14G8 bright" and "$14G8^-$" B cells was found in their differential sensitivity to activation by LPS. Experiment I, Table III demonstrates this point by showing a 2-fold enrichment in LPS-responsiveness of "14G8 bright" cells and a 2-fold reduction in LPS-stimulated proliferation of "$14G8^-$" cells in comparison to responses of unseparated cells. Data presented in experiment II show a more dramatic 3.5-fold enrichment and 5-fold reduction in LPS-responsiveness of "14G8 bright" and "$14G8^-$" B cells, respectively. It is unlikely that the poor responsiveness of $14G8^-$ cells to LPS can be explained by depletion of macrophages from this population. First, the addition of irradiated spleen cells to "$14G8^-$" cells does not enhance their responsiveness to LPS (data not shown). Second, "$14G8^-$" cells cultured without exogenous macrophages respond well to anti-μ.

These results were surprising in 2 respects. First, it was anticipated that "14G8 bright" cells, since they appeared to be analogs of B cells from defective mice, would respond poorly or not at all to anti-μ; second, we expected that "$14G8^-$" cells would be at least as responsive as "14G8 bright" cells to LPS, based on the finding that B cells from normal mice generally respond more vigorously to LPS than do B cells from defective mice.

Cell cycle analysis of sorted "14G8 bright" and "$14G8^-$" B cells. Since ^{3}H-thymidine incorporation is a measurement of the average rate at which responsive cells are synthesizing DNA, it does not accurately measure the net fraction of cells responding by DNA synthesis. In order to ascertain the fraction

TABLE II

Ontogeny of cells expressing the determinant recognized by 14G8 in spleen

Age	% B Cells[a]	% Cells Bound[b] by 14G8
1 wk	48	55
2 wk	54	37
6 wk	62	27
9 wk	55	25
3 mo	51	22
6 mo	52	20

[a] DCF_1 δ mice of indicated ages were sacrificed and their spleen cells were analyzed by FMF for percentage of B cells after incubation with a monoclonal rat anti-mouse IgM hybridoma supernatant followed by fluorescein-conjugated SJL anti-rat IgG.

[b] Percentage of cells expressing the determinant recognized by 14G8 was determined by FMF after incubation of cells with 14G8 hybridoma supernatant followed by fluorescein-conjugated SJL anti-rat IgG.

TABLE III

Mitogen responses of "14G8 bright" and "14G8 negative" B cells

	Responses to Anti-μ[a]								
	Expt. I			Expt. II			Expt. III		
	Medium only	Anti-μ[b]	Δ cpm	Medium only	Anti-μ	Δ cpm	Medium only	Anti-μ	Δ cpm
	cpm								
Fillers only[c]	168	351	183	658	N.D.[e]	—	531	944	413
Unsorted	1,751	18,796	16,045	3,445	15,259	11,814	1,981	23,434	21,453
14G8 bright	7,579	7,330	0	5,157	14,312	9,155	3,967	15,299	11,332
14G8 negative	1,305	21,182	19,877	2,321	16,710	14,389	1,669	22,562	20,893

	Response to LPS[d]				
	Expt. I		Expt. II		
	Medium only	LPS[e]	Medium only	LPS	Δ cpm
	cpm		cpm		
Unsorted	N.D.[e]	34,820	288	31,945	31,657
14G8 bright	N.D.	61,569	653	104,074	103,421
14G8 negative	N.D.	15,624	149	5,927	5,778

[a] All cultures contained 50,000 unsorted cells or FACS II-sorted cells.

[b] Anti-μ was used at a final concentration of 50 μg/ml.

[c] Cultures receiving anti-μ as a mitogen also contained 2×10^5 CDF_1 δ spleen cells as fillers.

[d] LPS was used at a final concentrations of 50 μg/ml.

[e] Not determined.

of "14G8 bright" and "14G8⁻" cells synthesizing DNA in response to LPS and anti-μ, cell cycle analyses on stimulated cells were performed (Table IV). Cells were cultured at relatively high cell density (250,000 cells/0.2 ml/well) in order to avoid the need to add CDF$_1$ δ spleen cells as fillers. One day after initiation of cultures, colcemid was added to block cell division. Cell cycle analysis on viable cells was performed 2 days after initiation of cultures. Of the "14G8 bright" cells, 38% and 39% entered S phase in the presence of anti-μ and LPS, respectively, whereas only 5% of unstimulated "14G8 bright" cells entered S phase. In 2 separate experiments, approximately 40% of "14G8⁻" B cells entered S phase in the presence of anti-μ, but only 9.5% of "14G8⁻" cells cultured with LPS entered S phase. The background response without added mitogen was approximately 3% for "14G8⁻" cells. With background values subtracted (Table IV, Expt. II), 17% of unsorted cells, 33.5% of "14G8 bright" cells, and 7.2% of "14G8⁻" cells responded to LPS. Clearly, "14G8 bright" cells were enriched for LPS-responsive cells and "14G8⁻" cells were depleted of LPS-responsive cells. A similar analysis with cultures stimulated by anti-μ (Table IV, Expt. II) yielded rather similar percentages of each cell type (47.4% for unsorted cells, 32.19% for "14G8 bright" cells, and 39.99% for "14G8⁻" cells) responding by entering S phase.

LPS-responsiveness of plate-fractionated 14G8⁺ and 14G8⁻ B cells. Since a larger number of B cells are required for detailed kinetic analysis of mitogen responses than can be conveniently obtained by cell sorting, we attempted to use culture dishes sensitized with 14G8 hybridoma reagent for purification of 14G8⁺ and 14G8⁻ B cells. Typically, this type of fractionation procedure yielded B cells that were 7 to 10-fold depleted of 14G8⁺ cells and B cells that are 1.5- to 2-fold enriched for 14G8⁺ cells. Employing such "14G8-depleted" or "14G8-enriched" B cell preparations, we examined the kinetics of LPS-stimulated proliferation at 4 different cell densities (Fig. 6). Preparations of B cells depleted of 14G8⁺ cells responded very poorly to LPS when compared to unseparated cells. This difference in LPS responsiveness is most striking when cultures were pulsed with ^{3}H-thymidine on day 1. Since the slopes of the cell density-response relationship of the 3

TABLE IV

Cell cycle analysis of mitogen-stimulated "14G8 bright" and "14G8⁻" B cells

Expt. No.	B Cells[a]	Mitogen[b]	Cell Cycle[c]			
			$G_0 + G_1$	S	$G_2 + M$	$S + G_2 + M$
I	"14G8⁻"	Anti-μ	59.5	23.7	16.7	40.4
	"14G8⁻"	LPS	90.5	5.1	4.3	9.4
	"14G8⁻"	None	96.0	2.9	1.0	3.9
II	"14G8⁻"	Anti-μ	57.8	26.2	16.0	42.2
	"14G8⁻"	LPS	90.5	5.8	3.7	9.5
	"14G8⁻"	None	97.7	1.9	0.4	2.3
	"14G8 bright"	Anti-μ	62.2	19.0	18.8	37.8
	"14G8 bright"	LPS	60.8	24.7	14.6	39.3
	"14G8 bright"	None	94.3	4.0	1.7	5.7
	Unsorted	Anti-μ	49.9	30.1	20.0	50.1
	Unsorted	LPS	80.3	10.2	9.5	19.7
	Unsorted	None	97.3	1.7	1.0	2.7

[a] DFC$_1$ δ spleen cells were treated with anti-Thy1.2 + C and stained with fluorescein-conjugated 14G8. The brightest 10% of the stained cells were sorted as "14G8 bright" cells and the least fluorescent 40% of cells were sorted as "14G8⁻" cells. Single cultures containing 250,000 cells/well in a final volume of 0.2 ml were set up in 96-well microtiter plates. No CDF$_1$ δ filler cells were added.

[b] Affinity purified anti-μ and LPS were each used at a final concentration of 50 μg/ml.

[c] Cell cycle analyses were performed 44 to 46 hr after initiation of cultures. Colcemid was added to all cultures 24 hr after initiation of cultures to prevent activated cells from division.

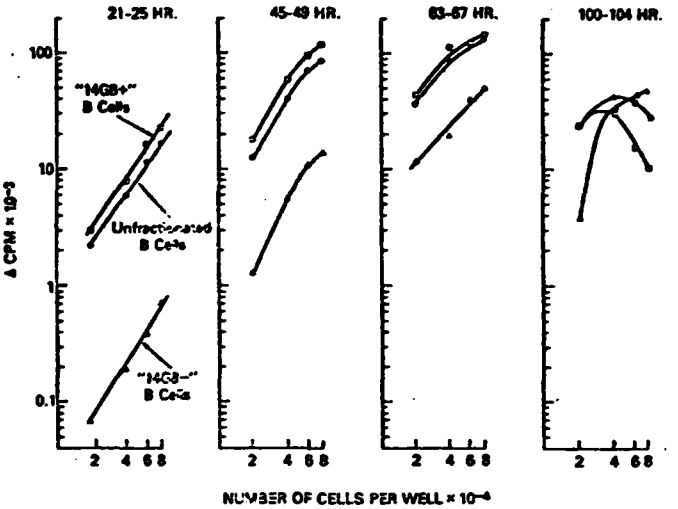

Figure 6. Kinetics of LPS-stimulated proliferation with plate fractionated subpopulations of B cells. Anti-Thy1 + C-treated DCF$_1$ δ spleen cells were fractionated on 14G8 sensitized Petri plates according to *Materials and Methods*. LPS induced proliferation is as follows: ●, unfractionated cells; □, "14G8-enriched" or "14G8⁺" cells; △, "14G8-depleted" or "14G8⁻" cells.

FMF analysis of plate fractionated cells showed that unfractionated cells contained 89.6% IgM⁺ cells and 41.4% 14G8⁺ cells; "14G8⁺" cells contained 97.1% IgM⁺ cells and 54.7% 14G8⁺ cells; and "14G8⁻" cells contained 73.0% IgM⁺ cells and 4.7% 14G8⁺ cells. Cultures were pulsed at indicated times with ^{3}H-thymidine.

populations of cells are very similar, one can reach the conclusion that "14G8-enriched" B cells respond approximately 10 times better to LPS than preparations depleted of 14G8⁺ cells. Since "14G8-enriched" B cells contained 97% B cells and "14G8-depleted" B cells contained 73% B cells, one could expect no more than a 30% difference in responsiveness based on the net number of IgM⁺ cells used.

Although differences between "14G8-enriched" cells and "14G8-depleted" cells are less striking, when the cells were pulsed with ^{3}H-thymidine on days 2 and 3, the 4-fold difference observed is still much higher than could be accounted for by the 30% difference in percentage of B cells. It is not surprising that "14G8-enriched" cells showed only a modest increase in LPS-stimulated proliferation in comparison to unstimulated cells, since 41.4% of unfractionated cells were 14G8⁺ and 54.7% of "14G8-enriched" cells were 14G8⁺.

DISCUSSION

A monoclonal rat antibody, 14G8, that recognizes a determinant expressed by a subpopulation of B cells, is described in this paper. It reacts with 30 to 40% of splenic B cells from immunologically normal DCF$_1$ δ mice and with approximately 65% of splenic B cells from CDF$_1$ δ mice, which express the *xid*-determined immune defect.

More than 90% of spleen cells expressing the determinant recognized by 14G8 are B cells, and such cells from DCF$_1$ δ mice are similar to unseparated CDF$_1$ δ B cells in having a relatively high median IgM density and a characteristic IgM staining pattern. "14G8⁻" B cells from DCF$_1$ δ mice bear low amounts of IgM, and this subpopulation of B cells appears to be missing or present in low numbers in CDF$_1$ δ mice.

In addition to its recognition of a subpopulation of B cells, 14G8 also shows weak reactivity to mouse erythrocytes, about 3% of nucleated bone marrow cells, and 50% of resident peritoneal washout cells. It does not show any reactivity toward thymocytes and peripheral T cells. The percentage of cells bearing the antigen recognized by 14G8 in neonates is high (approximately 50% at 1 wk of age) and steadily decreases to

about 20 to 25% of total spleen cells by 2 mo of age.

Ledbetter and Herzenberg (16) have described a rat monoclonal antibody 30-E2 which has tissue specificity similar to 14G8. Recently, Lanier *et al.* (30) have found 30-E2 to react with some but not all B cell lymphomas. We were able to block the binding of fluoresceinated 14G8 to spleen cells with 30-E2-containing hybridoma supernatant. This suggests that 30-E2 and 14G8 may be directed against the same antigenic determinant.

Mitogen responses as measured by ^{3}H-thymidine incorporation of sorted "14G8$^-$" and "14G8 bright" B cells from DCF$_1$ ♂ mice show that "14G8 bright" B cells are highly enriched in LPS-responsive cells, while "14G8$^-$" B cells are greatly depleted in LPS-responsive cells. However, both subpopulations of B cells are capable of responding to anti-μ by proliferation. Incubation of whole spleen cells with 14G8 did not alter their proliferative responses to LPS and anti-μ. Cell cycle analysis of mitogen-stimulated "14G8$^-$" and "14G8 bright" B cells also yielded similar results. Thirty-three percent of "14G8 bright" cells and only 7% of "14G8$^-$" B cells respond to LPS by DNA synthesis (enter S phase of cell cycle). With anti-μ as a mitogen, 38% of "14G8 bright" B cells and 40% of "14G8$^-$" B cells respond by synthesizing DNA. Background percentages of cells synthesizing DNA without added mitogen vary between 3 and 5%.

Dual parameter fluorescence analysis of spleen cells stained with fluorescein-conjugated 14G8 and biotin-conjugated anti-μ followed by XRITC-avidin showed that 90% of spleen cells expressing the determinant recognized by 14G8 are B cells (i.e., IgM$^+$ cells). The minor population of 14G8$^+$IgM$^-$ cells may be accounted for by: 1) cells with high background fluorescence that are artifactually scored as 14G8$^+$ cells even though they lack the antigen recognized by 14G8; 2) normoblasts, since 14G8 reacts with mature erythrocytes and might react with normoblasts, the precursors of the erythrocytes; 3) macrophages, since peritoneal macrophages express the antigen recognized by 14G8. Nevertheless, the vast majority (>90%) of spleen cells expressing the antigenic determinant recognized by 14G8 are B cells and among surface IgM$^-$ cells (including T cells and null cells), there are very few (<2%) that are recognizable by 14G8.

Because 14G8 reacts with a subpopulation of B cells in normal mice and because mice carrying the *xid*-determined immune defect are believed to be deficient in a subpopulation of B cells (1, 2), we examined the percentage of cells expressing the antigen recognized by 14G8 in an *xid* hemizygous mouse, the CDF$_1$ male. Even though phenotypically normal DCF$_1$ ♂ mice have a higher percentage of B cells among their spleen cells than do the defective CDF$_1$ ♂ mice (63 *vs* 43%), a higher percentage of spleen cells express the antigen recognized by 14G8 in the defective mice than in the normal mice (38 *vs* 22%). Furthermore, the vast majority (83%) of 14G8$^+$ cells from defective mice are IgM$^+$ and few, if any, cells among the IgM$^-$ cells express the antigenic determinant recognized by 14G8.

The subpopulation of B cells expressing the antigen recognized by 14G8 is similar to B cells from mice with the *xid*-determined defect in that both display heterogeneous IgM staining profiles (Fig. 5). In addition, 14G8$^+$ cells are very frequent among neonatal cells, and the percentage of B cells expressing the determinant recognized by 14G8 falls as the animal matures. This is the reverse of the ontogeny of Lyb-5$^+$ cells, which increase in frequency as the mouse matures (4). Since Lyb-5$^+$ B cells are absent in "*xid*" mice, it is possible that 14G8$^+$ B cells are Lyb-5$^-$. Indeed, preliminary experiments performed in our laboratories have shown that sorted "14G8 bright" B cells are not lysed by anti-Lyb-5 + C.

Because of the apparent similarity of 14G8$^+$ B cells from normal mice to B cells from mice with the *xid*-determined defect, one might expect 14G8$^+$ B cells to behave like B cells from defective mice in being unable to mount a proliferative response to anti-μ and in having a low-level background proliferation. However, contrary to expected results, we found 14G8$^+$ B cells to be responsive to anti-μ stimulated proliferation. Furthermore, 14G8$^+$ B cells display a much higher level of background proliferation than B cells from mice with the *xid*-determined defect.

CDF$_1$ ♂ B cells have also been reported to respond poorly to LPS at low cell concentrations (31). Thus, one might expect 14G8$^-$ B cells to be enriched in LPS-responsive cells. On the contrary, we found that 14G8$^+$ B cells were enriched in LPS-responsive cells and 14G8$^-$ B cells were severely depleted of LPS-responsive cells. Therefore, despite similarities between 14G8$^+$ B cells from normal mice and B cells from "*xid*" mice in expression of amounts of membrane IgM, the antigen recognized by 14G8, and in the lack of Lyb5 expression, the activation requirements of these cells appear to be very different.

Bone marrow B cells display a heterogeneous IgM staining pattern similar to that of cells from "*xid*" mice and of neonatal B cells. This suggests that B cells in the bone marrow may be comprised of mostly "immature" or neonatal-like B cells. If one associates the expression of the antigen recognized by 14G8 with "immature" B cells, it is difficult to explain the lack of expression of this antigenic determinant on most if not all bone marrow B cells. With presently available information, we could not decisively associate B cells from various tissues expressing or lacking the antigen recognized by 14G8 with discrete stages of B lymphocyte differentiation.

The key to understanding the relationship between the 2 subpopulations of B cells defined by 14G8 relies on the availability of large numbers of purified 14G8$^+$ and 14G8$^-$ B cells. We have recently been able to obtain preparations of large numbers of B cells enriched or depleted for cells expressing the antigen recognized by 14G8 using petri plates sensitized with 14G8. Results using plate-fractionated cells showed that they are very similar to FACS-sorted cells, as measured by proliferative responses to LPS as well as to anti-μ. Studies aimed at dissecting the relationship between the 2 subpopulations of B cells defined by 14G8, their functional properties, as well as their roles in B lymphocyte differentiation are now in progress.

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